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In this study, we are investigating the role of AP-1 in controlling breast cell growth and transformation. We have previously demonstrated that normal human breast cells have high basal levels of AP-1 activity and that breast cancer cells express relatively low levels of AP-1 activity. We have also shown that AP-1 complexes are activated by peptide and steroid growth factors in normal and malignant breast cells. In this report, we now show that other peptide growth factors, including IGF-1, EGF, heregulin-β, and FGF, stimulate AP-1 activity in breast cancer cells. This growth factor-induced AP-1 activity can be suppressed by the expression of TAM67. The mitogenic pathways activated by serum and these growth factors depend on AP-1 to transduce proliferative signal. AP-1 blockade induced by the expression of TAM67 inhibits breast cancer cell growth mainly by delaying S phase entry, and inducing G1 cell cycle arrest. In the future, we will determine whether AP-1 blockade inhibits breast cell growth *in vivo* and whether AP-1 blockade prevents oncogene-induced transformation of breast cells. These results suggest that AP-1 is a promising target of future cancer therapeutic and preventive agents since blocking this critical transcription factor suppresses proliferation induced by multiple growth factors.

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INTRODUCTION

During the fourth year of the funding period we have continued to investigate the affect of AP-1 blockade on the growth of breast cancer cells and have determined the mechanisms by which AP-1 blockade inhibits the growth of breast cells. By using our inducible MCF7-Tet-Off TAM67 cell lines, we demonstrated that TAM67 inhibits basal AP-1 activity and AP-1 activity stimulated by several different growth factors. We have also discovered that AP-1 activation is required to transduce growth factor-induced mitogenic signals in breast cells. These studies showed that inhibition of AP-1 completely blocked proliferation of MCF7 cells in response to the serum and peptide growth factors (IGF-1, EGF, heregulin-ß, and FGF). These results demonstrate that the mitogenic pathways activated by serum, IGF-1, EGF, heregulin-ß, and FGF depend on AP-1 to transduce a proliferative signal. We then determined the mechanisms by which TAM67 inhibits breast cancer cell growth. These studies showed that AP-1 blockade induced by the expression of TAM67 causes a G1 cell cycle arrest. In the absence of serum, TAM67 also causes apoptotic cell death. In addition, we have produced the TAM67 retroviral and adenoviral constructs. In the next year, we will utilize these reagents to investigate the role of AP-1 transcriptional activity in oncogene-induced transformation of breast cells and to reverse the oncogene-transformed phenotype of breast cells.

These studies have demonstrated that AP-1 is a critical regulator of breast cell growth. The results from these studies will provide the foundation for our ongoing efforts to develop agents that interfere with AP-1 signaling pathways. Such agents will likely be useful chemopreventive agents to block breast carcinogenesis.

BODY

BACKGROUND

Breast cancer is the most common malignancy in women, and the leading cause of death for women between the ages of 40 and 55 in this country (1). Even with aggressive mammographic screening, adjuvant chemotherapy, and intensive therapy for existing cancer, many of the women who develop breast cancer will die from it. Thus, more effective prevention strategies and treatments are urgently needed.

Unfortunately, little is known about the specific molecular events, which cause the progressive transformation of human breast epithelial cells to malignant breast cancer. Studies of model systems of cancer have revealed that multiple steps are involved in carcinogenesis, including tumor "initiation" and "promotion" events (2). Mutations and deletions within tumor suppressor genes may represent the molecular equivalent of breast cancer "initiation" events (3,4). However, the molecular mechanism of breast tumor "promotion" is poorly defined. In model systems (5), classic tumor promoters induce the proliferation of initiated cells, leading to the progressive outgrowth of fully malignant cells. Such tumor promoters typically activate signal transduction pathways to stimulate cellular proliferation. In human breast cells, the overproduction of growth factors, or aberrant stimulation of growth factor receptors, may be responsible for the promotional phase of breast carcinogenesis. Growth factors important for mammary epithelial cells, such as estrogen, EGF, TGF-a, and the IGFs, may all represent tumor promoters of human breast cancer. Thus, drugs which inhibit the ability of estrogen to activate the estrogen receptor (tamoxifen and other antiestrogens) are used to treat breast cancer. Other drugs which block growth factor receptors, such as antibodies specific for the epidermal growth factor receptor and the Her2/neu receptor, have been shown to inhibit breast cancer cell proliferation (9-11), and are now being tested in clinical trials for the treatment of breast cancer. However, inhibition of individual signal transduction pathways may ultimately be ineffective, since multiple different signal transduction pathways can stimulate breast cell proliferation. It may be more effective to inhibit signal transduction at a more distal point in the cascade, where multiple mitogenic signals converge. Since transcription factors, the nuclear proteins that control DNA transcription and gene expression, are the most distal components of these converging mitogenic signal transduction pathways, they may be ideal targets for new therapeutic agents.

A key family of transcription factors that transduces multiple mitogenic signals is the AP-1 family. These transcription factors are complexes of DNA-binding proteins made up of dimers of Jun and Fos proteins, which bind DNA at specific AP-1 sites and regulate the transcription of AP-1-dependent genes. AP-1 transcription factors are expressed in most cell types, and are activated by specific kinases, such as the mitogen-activated and stress-activated kinases, which are themselves activated by diverse signals such as growth factor stimulation, exposure to UV light, oxidative stress, tumor promoters such as TPA, or oncogene overexpression or activation (reviewed in 6).

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Thus, AP-1 is a central component of many signal transduction pathways in a variety of cell types.

Previous studies showed that the AP-1 transcription factor family is critical for growth factor induced proliferation of fibroblasts (12,13). In addition, we (14,15) and others (16) have shown that AP-1 is also critical for oncogene-induced transformation of fibroblasts. Specifically, we have demonstrated that AP-1 is critical for the cotransformation of primary rat embryo cells by *ras+jun*, *ras+fos*, or *ras+SV40 T antigen* (14), while others have shown that AP-1 is critical for the transformation of NIH3T3 cells by single oncogenes such as *ras*, *raf*, *abl*, and *mos* (16). Thus, AP-1 is a central regulator of transformation as well as mitogenic signaling.

While the role of AP-1 has been extensively studied in fibroblasts, relatively few studies of the function of AP-1 have been performed in epithelial cells. Thus the exact role of this transcription factor family in controlling the proliferation and transformation of epithelial cells is not known. Previous studies from our lab and others have demonstrated that the Jun and Fos family members are expressed in human breast cancer cells, and are activated by a variety of important growth factors for these cells, such as EGF, TGFa, and the IGFs. Other studies have also suggested that hormones such as estrogens and retinoids can modulate AP-1 transcriptional activity in breast cells. More recent studies suggest that ER and AP-1 interact to regulate the expression of certain estrogen and/or tamoxifen regulated genes (17). AP-1 complexes may be involved in regulating transcription of the ER gene as well (18). These results suggest that the AP-1 complex may be involved in controlling proliferation of human breast cells. However, definitive studies demonstrating that AP-1 is critical for either breast epithelial cell proliferation or transformation have not been performed.

To address these questions, we have used the 184 series of normal human mammary epithelial cells (HMECs) isolated and characterized by Dr. Martha Stampfer (19). These cells were originally isolated from reduction mammoplasties of patients and have a normal karyotype, EGF receptors, and specific cytokeratins, suggesting that they are derived from the basal epithelial cells of the normal breast. These HMECs are primary cells, which will senesce after 15-20 passages. However, by exposing these primary HMECs to the carcinogen benzo(a)pyrene, Stampfer *et al.* (19) have established multiple immortalized lines of HMECs (the 184A1 and 184B5 lines). We are studying these carcinogen-immortalized cells as well as the spontaneously immortalized HMEC line, MCF10A, derived from breast tissue obtained from a patient with multiple fibrocystic nodules (20). This cell line expresses cytokeratins and epithelial mucins consistent with a breast epithelial origin, and has cytologic characteristics of breast luminal ductal cells (21). None of the immortal cells are fully transformed since they are not able to grow in an anchorage-independent fashion, or form tumors in nude mice. Recent reports have demonstrated that these immortalized human mammary epithelial cells can be transformed by specific oncogenes such as activated *ras* (22, 23) or *erbB2* genes (24), or by overexpression of *c-myc* or *SV40 T* genes (16). In particular, MCF10A cells can be transformed by an activated *ras* gene (23), while 184B5 cells can

be fully transformed by activated *ras* genes (22), or by overexpression of *c-erbB2* (24). Many of these oncogenes are known to activate AP-1 in fibroblasts, though whether these oncogenes also activate AP-1 in breast epithelial cells is not yet known. If AP-1 is involved in regulating these processes, it might therefore serve as a target for the prevention or treatment of breast cancer. To determine the role of AP-1 in controlling breast cell growth and transformation, we proposed to test the following hypotheses:

1. Human breast epithelial cells at different stages in the carcinogenesis pathway express different levels of the AP-1 transcription factor.
2. Breast epithelial cells at these different stages have different requirements for AP-1 for their growth.
3. AP-1 transcription factor activity is necessary for *in vitro* transformation of human breast epithelial cells.

In our previous reports we demonstrated that AP-1 transcription factor expression and transcriptional activity is high in normal mammary epithelial cells and is progressively reduced as breast cells proceed towards malignancy. These studies were originally proposed in **Specific Aim 1**. We also previously reported data demonstrating that the growth of normal and immortal cells is suppressed by AP-1 blockade. These studies, proposed in **Specific Aim 2** of the original grant proposal, demonstrated that normal, immortal and some cancer cells depend on AP-1 to transduce mitogenic signals, and that normal cells are more sensitive to the AP-1 blockade than are breast cancer cells. Our results also suggest that mitogenesis induced by peptide growth factors, such as serum, EGF, IGF-1, is critically dependent on AP-1, while growth induced by estrogen is likely mediated partly through an AP-1-independent signal transduction pathway. We now report the results from our experiments performed over the previous year (the fourth year of this project). Studies described in this annual report demonstrate that the mitogenic pathways in MCF-7 breast cancer cells activated by heregulin-ß, FGF, also depend on AP-1 to transduce a proliferative signal. We also demonstrated that TAM67 inhibits breast cell growth by blocking the cell cycle. TAM67 induces a G1 cell cycle arrest. In addition, in the absence of serum, blockade of AP-1 by TAM67 induces apoptosis. To investigate whether AP-1 blockade suppresses the transformation of breast cells as described in **Specific Aim 3**, we have produced a TAM67 retrovirus and an adenovirus expressing TAM67. We are now using these reagents to complete the studies proposed in **Specific Aim 3**.

EXPERIMENTAL METHODS AND PROCEDURES

Primary Cell Cultures and Cell Lines:
Human mammary epithelial cells and cell lines used in these studies (Table 1) include normal HMECs isolated from epithelial organoids of human breast from Clonetics (passages 9-10); normal 184 cells (15); 184A1 and 184B5, nontumorigenic immortal cell lines derived from benzo(a)pyrene-treated 184 cells (19); MCF10A (from Dr. J. Russo), a nontumorigenic spontaneously immortalized HMEC cell line; MCF10AneoT

(from Dr. J. Russo, Fox Chase Cancer Center, Philadelphia, PA), a transformed cell line derived from MCF10A transfected by c-Ha-ras; MCF7 WT (wild-type), a human breast adenocarcinoma cell line; MCF7 Adria, a doxorubicin (Adriamycin)-resistant subclone of MCF7 WT (from Dr. K. Cowan, National Cancer Institute, Bethesda, MD). Cells were grown in the following culture media: MEGM (Clonetics, San Diego, CA) for normal HMECs 184, 184A1, and 184B5 (19,25); DME/F-12 with 5% horse serum and supplements as described (20, 23) for MCF10A and MCF10AneoT [with 400 μg/ml Geneticin (G418, Life Technologies, Inc., Gaithersburg, MD); and Improved MEM (high zinc option; Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin for MCF7 and MDA MB 435.

Plasmids:
To develop the pLPCX-TAM67 retroviral construct, the TAM67 fragment (0.6 kb) which is tagged with FLAG was released from pCDNA(-)3.1-FLAG-TAM67 with HindIII/XhoI. The fragment was re-ligated to the pLPCX (Clontech) retroviral vector. To produce the pCA14-TAM67 adenoviral construct, the TAM67 fragment (0.66 kb) which is tagged with FLAG was released from pCDNA(-)3.1-FLAG-TAM67 with EcoRI/HindIII. The fragment was then cloned into the pCA14 shuttle vector. This vector is being used to produce the TAM67 adenovirus.

Transfection of Breast Cells:
184, clone 91, 184B5, MDA MB 231, MCF7, and T47-D breast cells were transfected using Fugene 6 reagent (Boehringer Mannheim) while MCF10A and MDA MB 435 breast cells were transfected using the LT-1 transfection reagent (PanVera Corp.) according to manufacturer's recommendations.

Western Blot Analysis:
Equal amounts of total cellular protein extract were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad). Primary antibody used was rabbit anti-cJun Ab-1 from Oncogene Science (Cambridge, MA). Blots were developed using the enhanced chemiluminescence (ECL) procedure (Amersham).

Luciferase Assay to Measure AP-1 Activity:
AP-1 transcriptional activating activity in cells was measured using the Dual-Luciferase™ Reporter Assay (Promega) as previously described (13). The cells were co-transfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter which contains a single AP-1 binding site (TGAG/CTCA) between nucleotides –73 and –60 and pRL-TK. To determine the AP-1 activity stimulated by growth factors, the cells were treated with EGF (100 ng/ml), IGF-1 (100 ng/ml), herogulin-β1 (10 ng/ml) or b-FGF (10 ng/ml), respectively for 6 hours before harvest. Transfected cells were lysed 36 hours after transfection and luciferase activity was measured with equal amounts of cell extract and normalized with the Renilla activity.

Cell Growth Assays:

Cell proliferation assay of stably transfected Tet-On and Tet-Off cell lines
The CellTiter 96[TM] AQueous Non-Radioactive Cell Proliferation Assay (MTS assay; Promega, Madison, WI) was used to measure breast cancer cell growth according to the protocol provided by the manufacturer. 1000 to 2000 cells were seeded in a 96 well plate and doxycycline was added (MDA MB435 rtTA-vector or -TAM67 lines) or removed (MCF7 tTA-vector or -TAM67 lines) was added the next day and replaced every other day. A solution containing a 20:1 ratio of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium) and PMS (phenazine methosulfate) was added to the cells for 2 hours at 37° C and absorption at 495 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Cell proliferation assay of breast cells treated with specific growth factors:
The MTS assay described above was used to measure breast cancer cell growth after stimulation with specific growth factors, including heregulin-ß1 (0 to 10 ng/ml), b-FGF (0 to 10ng/ml. The cells were seeded in 24 well plates in full medium with doxycline (MCF7 cells) or without doxycycline (MDAMB435 cells). The cells were allowed to attach overnight, and then were washed and cultured in serum free medium (and in the case of estrogen treated cells, estrogen- and phenol red-free medium) for 48 hours. The media was then changed to doxycline-free media (MCF7 cells) or doxycycline-containing media (MDAMB435 cells), to induce TAM-67 protein, with different levels of the specific growth factors. The cells were then cultured at 37° C for 1 to 7 days. Cells were harvested every other day and the MTS assay was done as described above to measure proliferation. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

[3]H-Thymidine Incorporation Assay:
3×10^4 cells were seeded in a 24-well plate and then were starved in medium without any growth factors to synchronize the cells. The cells were then labeled with ^{3}H-thymidine (2uCi/ml) for 1 hour followed by incubated with 5% TCA at 4°C for 30 minutes. The cells were then lysed by addition of 0.1 N NaOH. 3H-thymidine uptake was measured by mixing the cell lysates with scintillation fluid and counting the ^{3}H cpm in a scintillation counter. Each data point was performed in sextuplet, and the results were reported as cpm +/- standard error. All results were normalized to cell numbers.

Flow Cytometry Assay:
MCF-7 Tet-Off TAM67 Clone #62 cells were kept in –Dox and +Dox media to induce or block the expression of TAM67. 24 hours before harvest, the medium is changed to serum-free medium to synchronize the cells. 2×10^6 cells were harvested at time point 0h, 6h, 12h, 24h, 48h, and fixed in 95% ethanol for 30 min in room temperature, and then stored in 4°C until ready to stain. For propidium iodide staining, the cells were pelleted and resuspended in 1 X PBS. 1 ml of 50 ug/ml of PI was used to stain the

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cells. Stained cells were analyzed with EPICS XL-MCL flow cytometer (Coulter Co.).
Histograms were then analyzed for cell cycle compartments.

Apoptosis Assay:
MCF-7 Tet-Off TAM67 Clone #62 cells were kept in –Dox and +Dox media to induce
or block the expression of TAM67. For TUNEL assay, the cells were trypinized and
pelleted. These samples were then embedded on agar, cut and mounted on slides and
processed for TUNEL assay as previously describled. For cell death ELISA assay, 1
$x10^4$ cells were seeded in 96 well plate overnight. This apoptosis assay measures
cytoplasmic DNA fragments and was performed according to the protocol of Cell
Death ELISA Kit (Roche). Each sample was performed in triplet and the results
expressed at mean +/- standard error from at least two independent experiments.

Retrovirus Preparation and Infection:
Phoenix ampho cells were maintained in Dulbecco's Modified Eagle Medium supplied
with 10 % of fetal bovine serum, 1 % of penicillin/streptomycin and 1 % of L-
glutamine. 2.0×10^6 cells were seeded in a 60-mm dish 18-24 hours prior to
transfection. pLPCX-TAM67 and control plasmids were transfected with Fugene 6
Regent (Roche) according to manufacture's protocol. Chloroquine was used to
increase the yield of retrovirus. 48 hours post-transfection, the retroviral supernatants
were harvested and filtered through a 0.45 um filter, then kept in –70°C freezer for
future use. The titers of the retrovirus were determined by infecting NIH3T3 cells and
using limiting dilution according to the protocol from Clontech. For infection with
retrovirus, 5×10^5 cells were plated into 10 cm dish 12-18 hours prior to infection, the
cells were then treated for 6 hours or overnight with infection cocktail containing equal
amounts of retroviral supernatant and growth medium and 4 ug/ml polybrene. After
infection, the medium was changed to normal growth medium and the cells were
incubated for another 36 to 48 hours. pLEGFP-N1 retroviral supernatant was also
used as control.

Soft Agar Cloning :
Tp prepare plates for soft agar cloning, SeaPlaque Agarose (low gelling) was added to
the stock solution to produce a 3.5 % stock solution made with PBS. Pre-warmed
media was added to get a 0.7 % agarose. For the lower feeder layer, 1.5ml of 0.7 % of
agarose was added to 6-well plate and then allowed to solidify by incubating the plate
at 4°C for 20 to 30 min. The cells were trypsinized and a single cell suspension was
made. A 0.35 % solution of agarose was prepared by adding five thousand cells to 4
ml of agarose. 4ml of cell/agarose were plated into the 6-well plate with solidified
bottom layer. The plate was then placed into 4°C refrigerator for 1 to 2 hours to
solidify the upper layer and then transferred to 37°C incubator. The clones were
allowed to grow for 14 days and then were counted.

RESULTS

AP-1 Expression and Activity in Breast Cells:

In **Specific Aim 1** we proposed to determine whether changes in AP-1 expression or activity occur as breast cells progress through different stages of carcinogenesis. Breast cells used in this study are listed in Table 1. We have previously shown that normal human mammary epithelial cells have high basal AP-1 activity, immortal breast cells have an intermediate level of basal AP-1 activity, and breast cancer cells have low basal AP-1 activity. We described these results in the 1996-97 annual report and in a 1997 *Cancer Research* publication (Smith *et al.* 28).

Table 1: Breast cells used in this study.

Cells	Name	Source	Phenotype
Normal HMECs:	HMEC-91	Clonetics	Senescent, anchorage-dependent
	184	M. Stampfer	
Immortal HMECs:	184B5	M. Stampfer	Immortal, anchorage dependent
	MCF10A	A. Russo	
Breast Cancer cell lines:	MCF7 WT	K. Cowan	Cancer cells, anchorage-independent
	T47-D	ATCC	and tumorigenic
	MDA MB 231	ATCC	
	MDA MB 435	ATCC	

In **Specific Aim 2** we proposed to determine whether the growth of breast cells at different stages of tumorigenesis are differentially affected by inhibiting AP-1 activity. We have investigated the affect of AP-1 blockade on the growth of normal, immortal, and fully malignant breast cells. These studies have demonstrated that the growth of normal and immortal and some malignant cells is suppressed by AP-1 blockade, while the growth of some other breast cancer cells is not suppressed. These results were described in the 1998-99 annual reports and presented as abstract at the 2000 AACR Annual Meeting in San Francisco and in the submitted manuscript by Ludes-Meyers *et al.*. This manuscript was originally submitted to Cancer Research, but was not accepted for publication. We have revised the manuscript, and it has been submitted to Oncogene.

Tet-On And Tet-Off Inducible Breast Cancer Cells Expressing TAM-67:

We previously described the isolation of breast cancer cell lines that express TAM-67 under the control of Tet-inducible or Tet-repressible promoters. For these experiments described in this report, we used: MCF7-Tet-Off TAM-67 cells: These cells do not express TAM-67 when the cells are grown in doxycycline (1ug/ml), but do express TAM-67 protein when grown in the absence of doxycycline.

Effect of Inhibiting AP-1 Transactivating Activity on Proliferation Induced by Specific Growth Factors:

In our last year's annual report, we described that AP-1 blockade induced by overexpression of TAM67 inhibited MCF-7 cell growth. TAM67 also inhibited MCF-7 growth induced by EGF, IGF-1, and partially inhibited the growth induced by estrogen. Over the last year, we have investigated the effect of blocking AP-1 on mitogenesis induced by other growth factors. In these experiments, we found that TAM67 inhibited basal AP-1 activity and AP-1 activity stimulated by different growth factors (IGF-1, EGF, heregulin-β, and FGF) (Fig 1). MCF7 Tet-Off TAM-67 cells (Clone #62), or MCF7 Tet-Off vector cells (Vector Clone #1) were treated with different concentrations of heregulin-β (0, 0.1, 1.0, 10.0 ng/ml), or FGF (0, 1.0, 10.0, 100.0 ng/ml) in the presence and absence of doxycycline. When the TAM-67 MCF7-Tet-Off clone was grown in the presence of doxycycline (no TAM-67 expressed), these MCF-7 clones proliferated normally in response to serum stimulation. However, when doxycycline was withdrawn, TAM-67 was expressed, and growth factor-induced proliferation was inhibited (Fig 2, 3).

Mechanism by Which TAM67 Inhibits Breast Cancer Cell Growth:

We have previously demonstrated that AP-1 blockade inhibits normal and malignant breast cell growth. We predicted that AP-1 blockade causes either cell cycle block or apoptosis. To determine whether AP-1 blockade suppresses DNA synthesis, we used the MCF-7-Tet-Off-TAM67 cells under un-inducible and inducible conditions to measure ^{3}H-Thymidine uptake. The results demonstrated that the expression of TAM67 inhibits the DNA uptake of MCF-7 cells (Fig. 4). Then we performed flow cytometry assay under same conditions. The flow cytometry assay results showed that when Dox was induced, there were fewer cells in S phase and more cells in G0/G1 phase compared to cells were cultured in Dox-containing medium (Fig. 5). The results from ^{3}H-Thymidine uptake and flow cytometry are consistent, which demonstrate that the AP-1 blockade induced by the expression of TAM67 causes the G1 cell cycle arrest of MCF-7 breast cancer cells.

We then investigated whether AP-1 blockade induced apoptosis. Results from Cell Death ELISA Assay demonstrated that the expression of TAM67 did not induce apoptosis in MCF-7 cells when the cells were cultured in normal full medium (Fig 6). TUNEL results also confirmed these results. However, when the cells were cultured in serum-free medium, TAM67 caused apoptosis (Fig. 6). These studies demonstrated that AP-1 blockade induced by expression of TAM67 inhibit MCF-7 breast cancer cell growth mainly by inducing G1 cell cycle arrest.

The results of experiments investigating the mechanism by which TAM67 inhibits breast cell growth are included in a manuscript in preparation entitled "Growth inhibition effects of TAM67, a specific dominant negative inhibitor of cJun".

Suppression of Oncogene-induced Transformation by AP-1 Blockade:

In **Specific Aim 3** we proposed to determine whether inhibition of AP-1 activity prevents the *in vitro* transformation of immortalized breast cells. We have previously successfully transfected oncogenes, such as c-Ha-ras or c-erbB2, into immortal 184B5 and MCF10A cells and transformed these cells into cells that exhibit anchorage-independent growth. We are now using two different approaches to investigate whether TAM-67 blocks oncogene-induced transformation:

1). We are attempting reverse the phenotype of already transformed cells. For these experiments, we constructed pLPCX-TAM67 retroviral vector. We transfected the vector into Pheonix ampco package cells and harvested viral supernatant. We infected the Ras- and erbB2- transfected 184B5 and MCF10A cells with the virus. We will then measure the effect of AP-1 blockade on the anchorage independent growth of these cells in soft agar.

2). As an alternate strategy, we are constructing an adenovirus expressing TAM67. We will use this adenovirus to infect oncogene-transfected immortal breast cells. We will determine whether TAM67 reverses the anchorage independent growth phenotype.

From these experiments, we will determine whether AP-1 blockade prevents or reverses the transformed phenotype of oncogene-transformed cells.

If these studies demonstrate that TAM-67 blocks anchorage-independent growth, then in future studies we will investigate the effect of AP-1 blockade on *in vivo* tumorigenesis.

DISCUSSION

The data presented here, along with our previous data, demonstrate that AP-1 blockade induced by TAM-67 inhibits the growth of normal, immortal and some breast cancer cells (such as MCF7 cells), but that other breast cancer cells (such as MDA MB 435) are relatively resistant to AP-1 blockade. The present results also show that mitogenesis induced by individual peptide growth factors, such as IGF-1, EGF, heregulin-ß, and FGF, can be completely blocked by expression of TAM-67, while mitogenesis induced by estrogen is only partially inhibited by AP-1 blockade. These data suggest that peptide growth factor-induced mitogenesis is critically dependent on AP-1 in MCF-7 cells. The data also suggest that estrogen-induced mitogenesis is only partially dependent on AP-1 and that estrogen-induced signaling likely involved other AP-1 independent pathways. Our recent data also demonstrate that AP-1 blockade induced by expression of TAM67 causes growth inhibition by suppressing entry into S-phase of the cell cycle. In the absence of serum, expression of TAM67 also induces apoptosis.

Multiple growth factors have been shown to stimulate the proliferation or

differentiation of normal HMECs and breast cancer cells (15, 29,30). EGF stimulates the growth of normal HMECs (15) as well as breast cancer cells (29,30), and heregulin has been found to modulate the growth and differentiation of immortal HMECs (31). Other hormones that affect the growth of breast cancer cells include estrogen (32,33) and insulin-like growth factors (IGF-1 and IGF-2) (34), which induce proliferation, and retinoids, which inhibit proliferation and induce differentiation (35,36). Previous studies from our own lab have demonstrated AP-1 complexes are activated by important growth factors for breast cells, such as EGF, and IGFs (28). These previous results suggested that the AP-1 complexes might be involved in controlling proliferation of human breast cells. The current data now demonstrated that peptide growth factors stimulate breast cancer cell proliferation through the AP-1 signaling pathway. Thus, the AP-1 transcription factor is a critical signaling molecule in normal breast cells and in some breast cancer cells (i.e. MCF-7), but apparently not in other breast cancer cells (i.e. MDA MB 435).

C-Jun is a major component of the AP-1 transcriptional complex. AP-1 plays a critical role in cell's proliferation and transformation. For example, homozygous jun-/- mouse embryos die after 12-14 days at mid-to-late gestation (37, 38). In addition, cellular transformation induced by many oncogenes requires c-jun (39). AP-1 functions by regulating AP-1 dependent downstream genes, or by interacting with transcriptional co-activators, such as JAB1 (Jun activation domain binding protein 1), or integrators, such as CBP or p300 (40, 41, 42). However, AP-1 activated genes whose products participate in the progression from G1 to S phase are yet to be identified. Here we showed data demonstrating that AP-1 blockade induced by expression of TAM67 delays S phase entry and causes G1 cell cycle arrest. We are currently investigating the mechanism by which AP-1 blockade delays S phase entry.

Recently, it has been suggested that AP-1 components and their upstream kinases (mainly JNKs) may also be involved in apoptosis (43-45). Although much attention has been directed towards the possible role of AP-1 in the induction of apoptosis, it should be noted that AP-1 may play a totally opposite role, namely providing protective function in response to some stresses in some cell types (46-48). Thus, inhibition of AP-1 may induce apoptosis in some conditions. In our studies, AP-1 blockade induced by expression of TAM67 induces apoptosis in serum free medium, but not in the presence of serum.

We have previously demonstrated that normal human breast cells have high basal levels of AP-1 activity and that breast cancer cells express relatively low levels of AP-1 activity. We have also shown that AP-1 complexes are activated by peptide and steroid growth factors in normal and malignant breast cells. In this report, we now show that other peptide growth factors, including IGF-1, EGF, heregulin-β, and FGF, stimulate AP-1 activity in breast cancer cells. This growth factor-induced AP-1 activity can be suppressed by the expression of TAM67. The mitogenic pathways activated by serum and these growth factors depend on AP-1 to transduce proliferative signal, and that estrogen-induced growth is only partially inhibited by AP-1 blockade. AP-1 blockade

induced by the expression of TAM67 inhibits breast cancer cell growth mainly by delaying S phase entry, and inducing G1 cell cycle arrest. In the future, we will determine whether AP-1 blockade inhibits breast cell growth *in vivo* and whether AP-1 blockade prevents oncogene-induced transformation of breast cells. These results suggest that AP-1 is a promising target of future cancer therapeutic and preventive agents since blocking this critical transcription factor suppresses proliferation induced by multiple growth factors.

ONGOING STUDIES

We are continuing our studies of AP-1 inhibition in immortalized HMECs and breast cancer cells. During the following year of the grant we will:

Determine whether AP-1 blockade inhibits the transformed-phenotype of breast cancer cells and oncogene-transformed HMECs.

We proposed to determine whether inhibition of AP-1 activity prevent the *in vitro* transformation of immortalized breast cells. In our previous annual reports, we have described successful transfecting oncogenes, such as c-Ha-ras or c-erbB2 into immortal 184B5 and MCF10A cells. These transfected cells have been transformed into cells that grow in soft agar, unlike the parental immortal breast cells. We also prepared pLPCX-TAM67 retrovirus. We are now infecting the breast cancer cells and oncogene-transformed HMECs to determine the reverse effect of anchorage independent phenotype of TAM67 by doing soft agar assay. As an alternate strategy, we are preparing a TAM67-expressing adenovirus. We will do adenoviral infection of breast cancer cells and oncogene-transformed HMECs and do soft agar assay to determine the effect of TAM67 on anchorage independent growth of breast cells. The results of these experiments will allow use to determine whether AP-1 blockade prevents or reverses the transformed phenotype of oncogene-transformed cells.

KEY RESEARCH ACCOMPLISHMENTS AND REPORTABLE OUTCOMES

Specific Aim 1: To determine whether changes in AP-1 expression or activity occur as HMECs progress through different stages of carcinogenesis.
We have completed this specific aim and have completed tasks for months 1-36 listed in the statement of work.

Accomplishments:
- Our results demonstrated that Jun and Fos protein expression and AP-1 activity are high in normal human mammary epithelial cells, and are reduced as breast cells progress toward a more malignant phenotype

Reportable Outcomes:
The results of these studies were:
1. described in our first year annual report,
2. presented at the "Era of Hope" meeting in Washington, D.C., November, 1997,
3. published as an abstract in the Era of Hope meeting booklet, 1997
4. published in *Cancer Research* (28).

Specific Aim 2: To determine whether growth of HMECs at the different stages is differentially affected by inhibiting AP-1 activity.
The tasks for months 1-36 have been completed and summarized in our previous reports and in this report.

Accomplishments:
- These studies demonstrate that normal and immortal human mammary epithelial cells require AP-1 for their growth.
- We successfully established MCF7 Tet-Off-TAM67 and MDAMB435 Tet-On-TAM67 cell lines.
- We discovered that breast cancer cells, which have relatively low basal AP-1 transcriptional activity, are less sensitive to AP-1 blockade. Of the breast cancer cells tested, MCF7 cells were the most sensitive to the growth suppressive effect of the AP-1 inhibitor.
- We also found that AP-1 blockade completely inhibits proliferation of breast cancer cells in response to peptide growth factors (IGF-1, EGF, heregulin-ß, and FGF), but only partially inhibits estrogen-induced proliferation.
- We determine the mechanisms by which TAM67 inhibits breast cancer cell growth. TAM67 mainly induces G1 cell arrest, and causes apoptosis in serum-free condition.

Reportable Outcomes:
These results were:
1. presented at the "*Era of Hope*" meeting in Washingtion, D.C., in November 1997 and published as an abstract in the meeting booklet.
2. presented at the the San Antonio Breast Cancer Symposium in 1997 and 1998, and published as an abstract in *Breast Cancer Research and Treatment* (51, 52).
3. presented at the 91st *AACR* Annual Meeting, April, 2000, San Francisco as a poster and published as an abstract in Proceedings of the American Association for Cancer Research, 41:500,2000.

4. presented at the *"Era of Hope"* meeting in Atlanta, June 8-11, 2000 as a poster and published as an abstract in the meeting booklet
5. described in the manuscript submitted for publication in *Oncogene*.

Specific Aim 3: To determine whether inhibition of AP-1 activity can prevent the *in vitro* transformation of immortalized HMECs.
The tasks for months 1-36 have been completed and summarized in our previous reports and in this report.

Accomplishments:

- We have developed an *in vitro* transformation assay and have isolated oncogene-transformed HMECs and determined their transformed phenotype.
- We have established several clones of 184B5 and MCF10A cells that stably express an activated erbB2 oncogene or an oncogenic ras protein. All of these clones exhibit the transformed phenotype of anchorage independent growth.
- These transformed HMECs and breast cancer cells will be used to determine whether inhibition of AP-1 transcriptional activity reverses the transformed phenotype of breast cells.
- We developed TAM67 retroviral vector---pLPCX-TAM67 and currently are preparing a TAM67 adenoviral vector.

Reportable Outcomes:

1. We have derived oncogene-transformed 184B5 and MCF10A cell lines.
2. Constructed a pLPCX-TAM67 retrovirus (currently constructing a TAM67 adenovirus).

CONCLUSIONS

During the fourth year of the funding period, we investigated the effects of AP-1 blockade induced by TAM67 on breast cell growth induced by growth factors and determined the mechanisms by which TAM67 inhibits breast cancer cell growth. Our results demonstrated that expression of TAM67 inhibited basal AP-1 activity and AP-1 activity stimulated by growth factors, including EGF, IGF-1, heregulin-ß, FGF. Our data also demonstrated that AP-1 blockade inhibits MCF-7 cell growth stimulated by serum, EGF, IGF-1, heregulin-ß, FGF. These data suggest that peptide growth factor-induced mitogenesis is critically dependent on AP-1 in MCF-7 cells. In addition, we investigated the mechanisms by which AP-1 blockade induced by expression of TAM67 inhibits breast cell growth. The results demonstrated that TAM67 mainly induces G1 cell cycle arrest, and under serum-free condition, it causes apoptotic cell death to suppress MCF-7 cell growth. We also have developed TAM67 retrovirus and now making TAM67 expressing adenovius which can be used to infected breast cancer cells and oncogene-transformed HMECs to investigate whether AP-1 blockade suppresses the transformed phenotype of oncogene-transformed human breast cells.

These studies have demonstrated an involvement of AP-1 transcription complexes in regulating human breast cancer cell proliferation through different signaling pathways. The results from these studies will provide the foundation for future efforts to develop agents that interfere with AP-1 signaling pathways. Such agents may be useful chemopreventive agents to block breast carcinogenesis.

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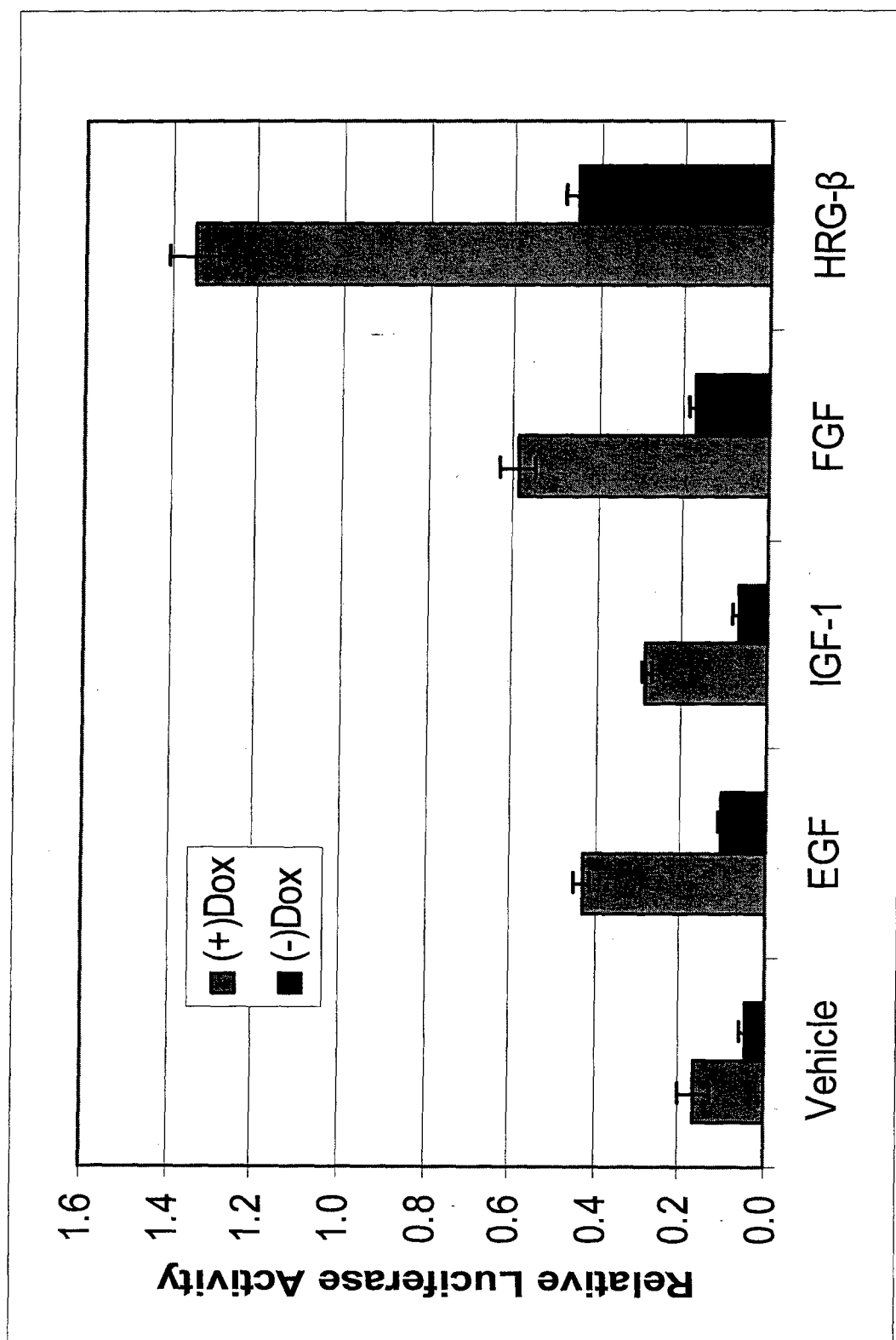
Figure 1: Blockade of AP-1 activity induced by different growth factors
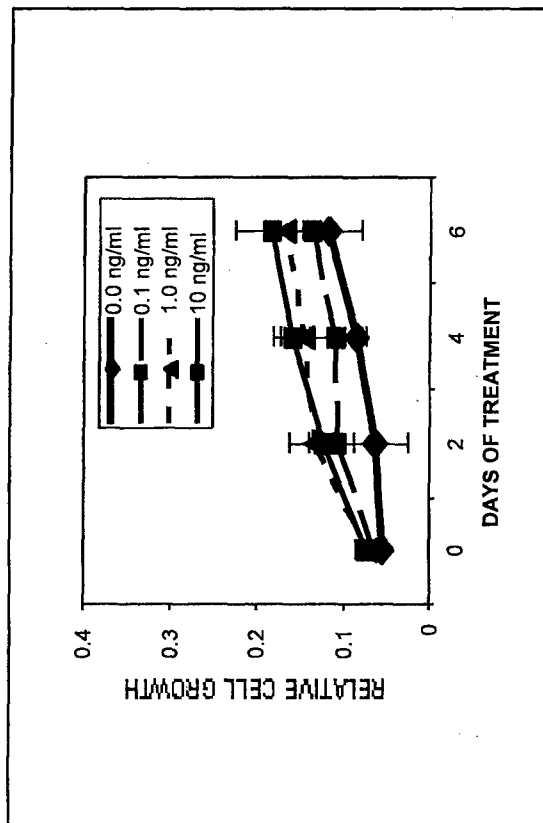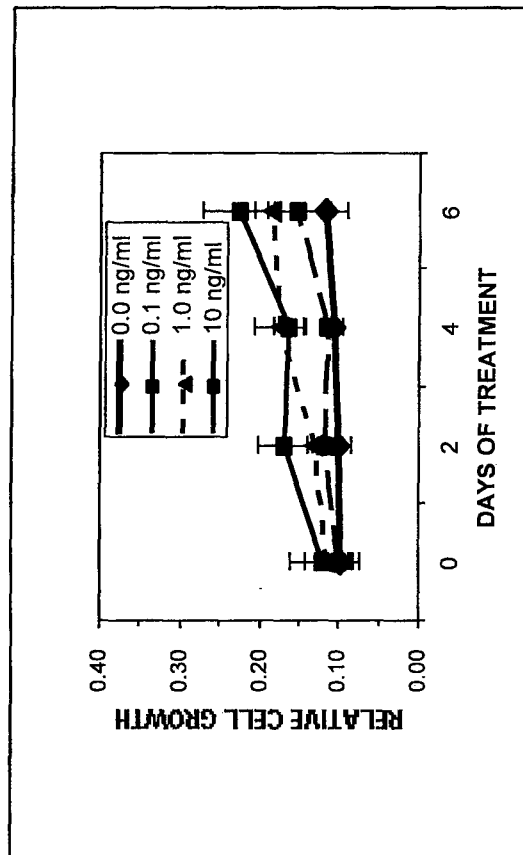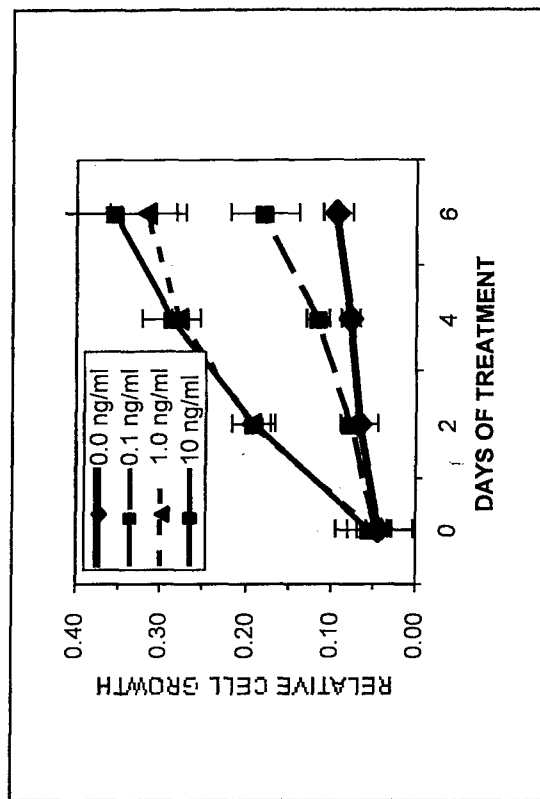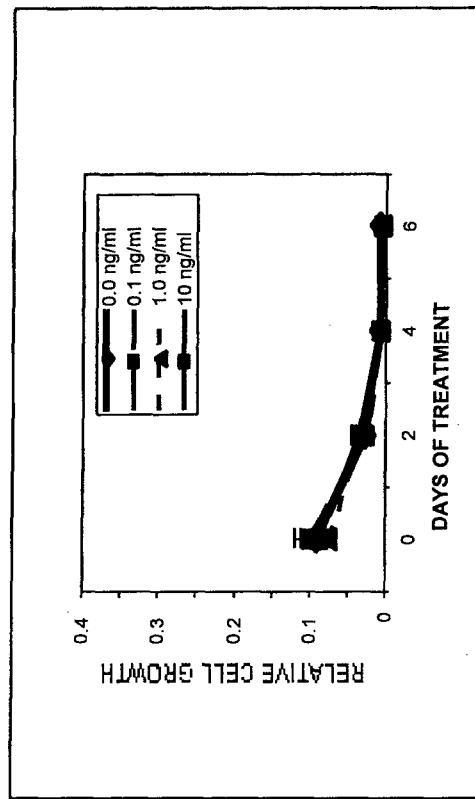
Figure 2: Effect of AP-1 blockade on heregulin-β-induced growth of MCF7
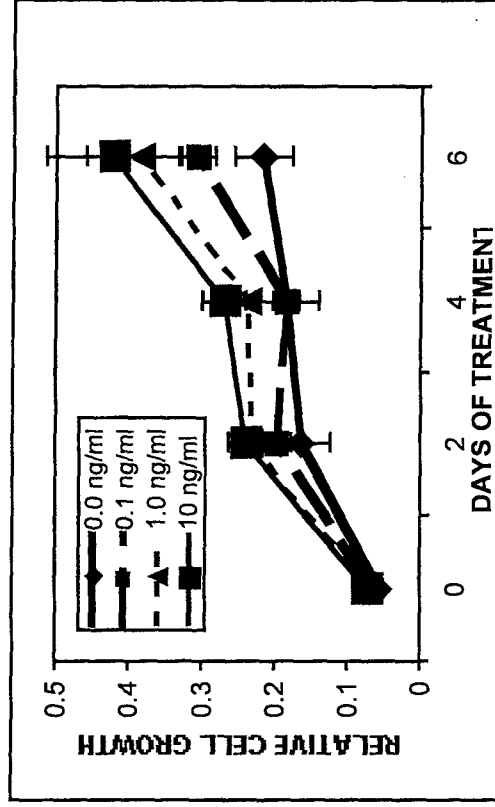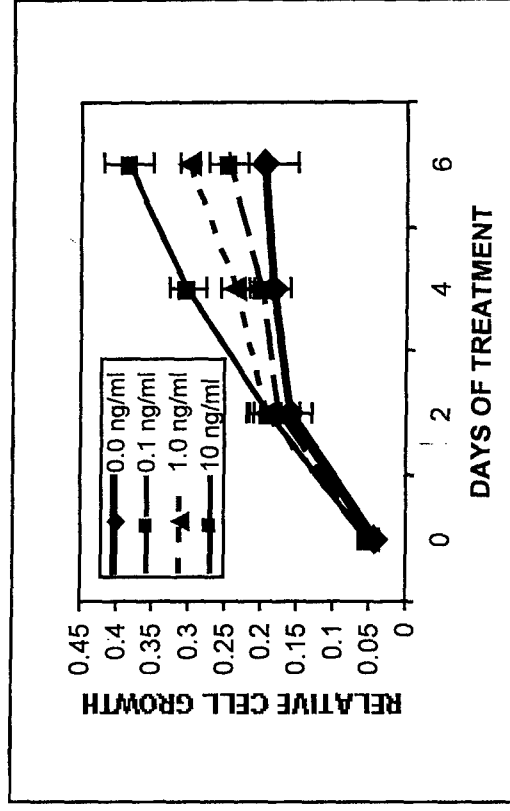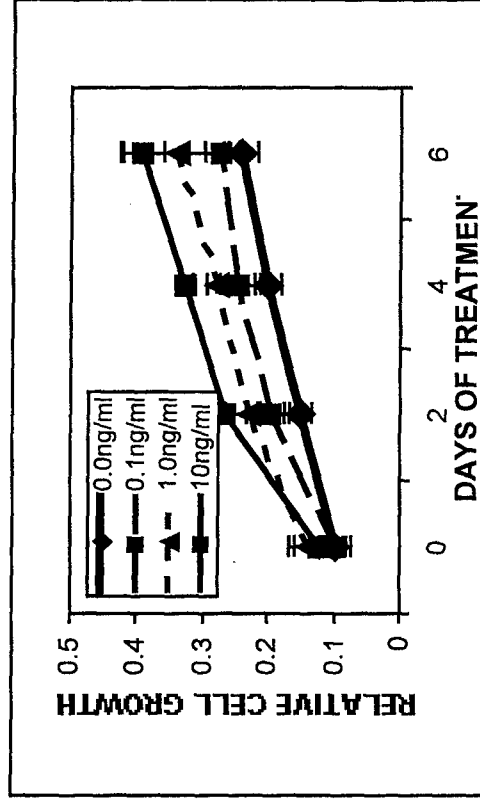
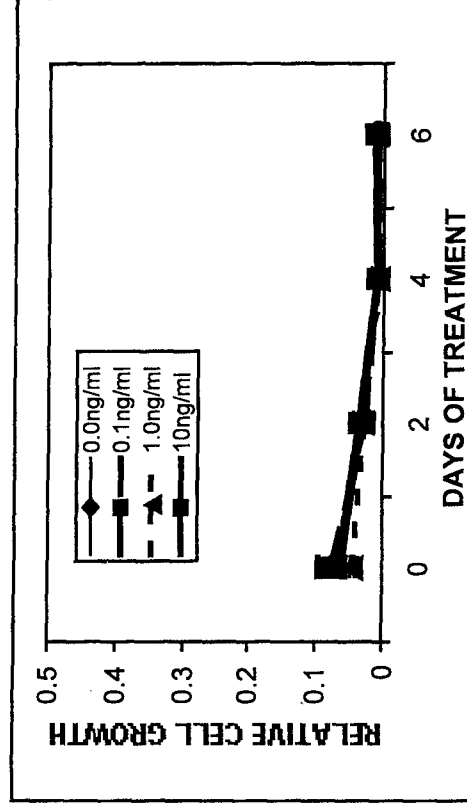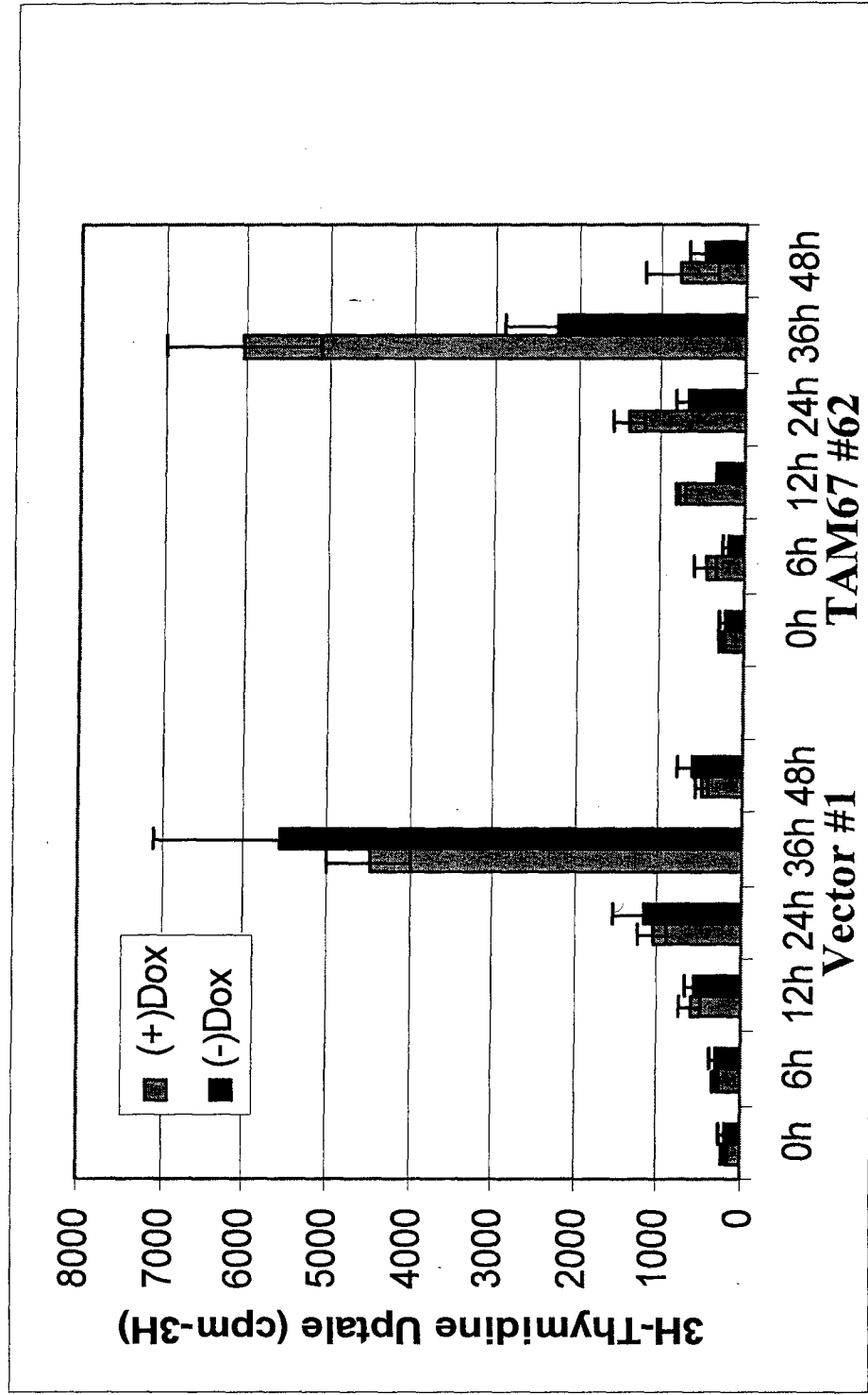Figure 3: Effect of AP-1 blockade on FGF-induced growth of MCF7 cells

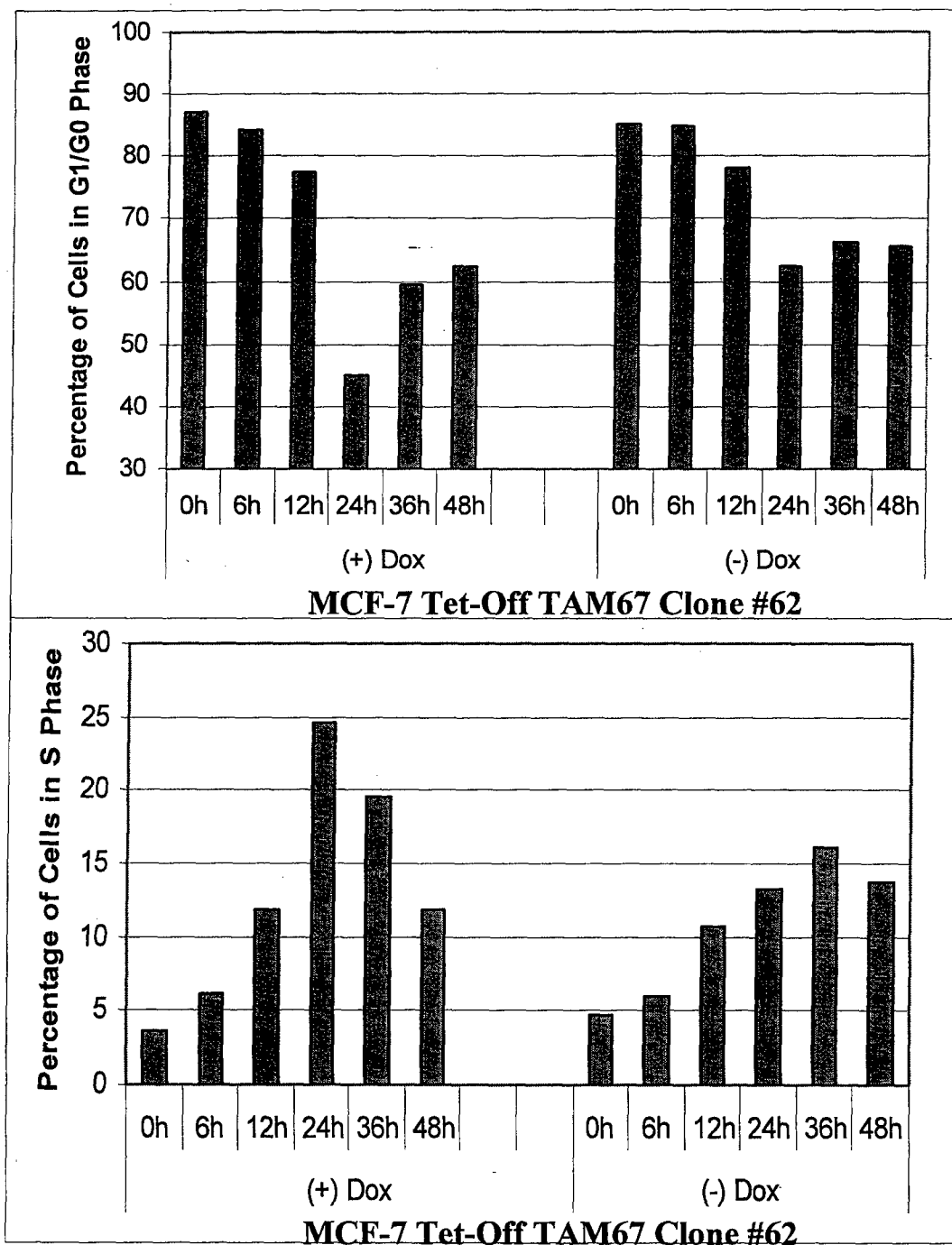Figure 4: Expression of TAN67 inhibits DNA uptake: 3H-thymidine Assay

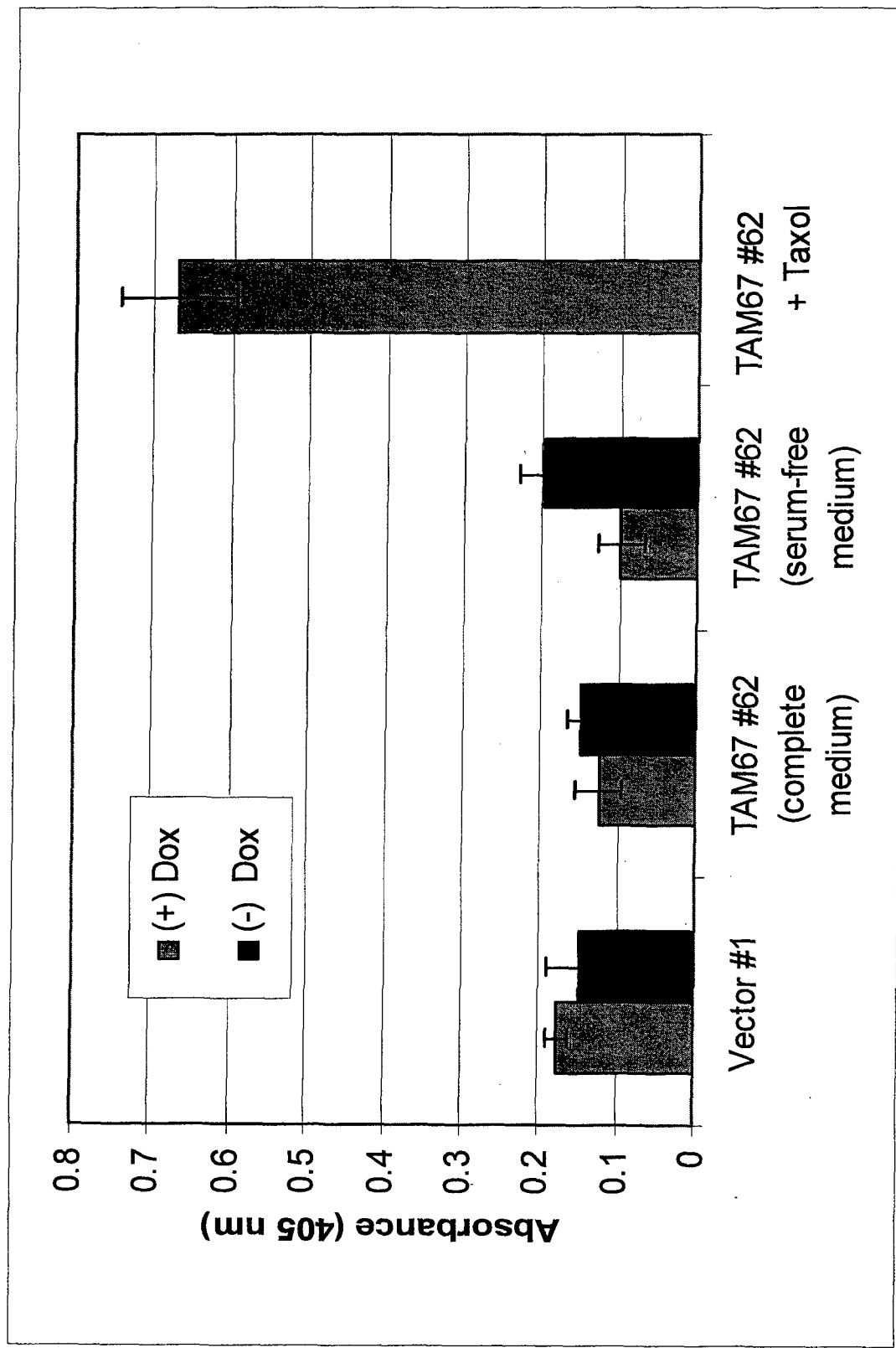Figure 5: Expression of TAM67 blocks normal cell cycle: Flow Cytometry Assay

Figure 6: Expression of TAM67 induces apoptosis in serum-free condition

Legend

Fig. 1 Blockade of AP-1 activity induced by different growth factor. MCF-7 Tet-Off TAM67 cells were kept in (+) Dox or (-) medium to block or induce the expression of TAM67. The cells were then transfected with Col-Z-Luc and pRL-TK. The cells were treated with EGF (100 ng/ml), IGF-1 (100 ng/ml), b-FGF (10 ng/ml), Heregulin-β1 (10 ng/ml), or vehicle for 6 hours, respectively. The AP-1 activity was measured using Dual-Luciferase Reporter System (Promega).

Fig. 2 Effect of AP-1 blockade on the heregulin-β induced proliferation of MCF-7 cells. MCF-7 Tet-Off TAM67 clone #62 cells and Vector clone #1 cells were grown in serum-free medium containing 0, 0.1, 1, or 10 ng/ml of heregulin-β1 under un-induced (+Dox) or induced (-Dox) conditions. Cells were harvested every other day and the MTS assay was done according to the protocol provided by manufacturer (Promega). Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Fig. 3 Effect of AP-1 blockade on the FGF induced proliferation of MCF-7 cells. MCF-7 Tet-Off TAM67 clone #62 cells and Vector clone #1 cells were grown in serum-free medium containing 0, 0.1, 1, or 10 ng/ml of b-FGF under un-induced (+Dox) or induced (-Dox) conditions. Cells were harvested every other day and the MTS assay was done according to the protocol provided by manufacturer (Promega). Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Fig. 4 Expression of TAM67 inhibits DNA uptake: ^{3}H-thymidine incorporation assay. MCF-7 Tet-Off TAM67 clone #62 cells and Vector clone #1were grown in (+) Dox or (-) Dox medium to block or induce the expression of TAM67. After synchronization in growth factor-free medium for 24 hours, the cells were labeled with ^{3}H-thumidine (2 μCi/ml) for 1 hour. The cells were lysed, and the ^{3}H-thymidine uptake was measured in a scintillation counter. Each data point was performed in sextuplet, and the results were reported as cpm +/- standard error.

Fig. 5 Expression of TAM67 blocks normal cell cycle: flow cytometry assay. MCF-7 Tet-Off TAM67 cells were grown in (+) Dox or (-) Dox medium to block or induce the expression of TAM67. 24 hours before harvest, the medium is changed to serum-free medium to synchronize the cells. The cells were harvested at time point 0 h, 6 h, 12 h, 24h, 48 h, and were stained with propidium iodide. Stained cells were analyzed with EPICS XL-MCL flow cytometer (Coulter Co.). Histograms were then analyzed for cell cycle compartments.

Fig. 6 Expression of TAM67 induces apoptosis in serum-free condition. MCF-7 Tet-Off TAM67 clone #62 cells and Vector clone #1were grown in (+) Dox or (-) Dox medium to block or induce the expression of TAM67. The experiment was carried out in complete medium and serum free medium. This apoptosis assay measures cytoplasmic DNA fragments and was performed according to Cell Death ELISA Kit (Roche). Each sample was performed in triplets and the results was expressed at mean +/- standard error.

ABBREVIATIONS

AP-1	Activating Protein 1
ATCC	American Type Culture Collection
ATF	Activating Transcription Factor
bp	base pairs
CBP	Creb Binding Protein
cDNA	complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced Chemiluminesence
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ERE	estrogen responsive element
FCS	Fetal Calf Serum
FGF	fibroblast growth factor
Ha-ras	Harvey-ras
HMEC	Human Mammary Epithelial Cells
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like growth factor binding protein
MAPK	mitogen activated protein kinase
MEGM	Mammary Epithelial Growth Medium
MEM	Modified Eagle Medium
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
neo	neomycin transferase
nm	nanometer
O.D.	Optical Density
PMS	phenazine methosulfate
rtTA	reverse tetracycline-controlled transactivator
SEM	Standard Error of the Mean
SV40	Simian Virus 40
tTA	tetracycline-controlled transactivator
Tet	Tetracycline
TetRE	Tetracycline Response Element
TGF	Transforming Growth Factor
TKR	tyrosine kinase receptor
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TRE	TPA responsive element
TUNEL	TdT (terminal deoxynucleotidyl transferase)-mediated dUTP-x nick end labeling
WT	Wild-type